Insulin secretion in the conscious mouse is biphasic and pulsatile

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INSULIN SECRETION OCCURS IN A BIPHASIC MANNER in response to rapid increases in glucose concentration (2, 8, 20, 45). First-phase secretion consists of a sharp peak that occurs within 2–5 min of a glucose challenge (29). It is generally accepted that the first-phase peak results from the glucose stimulus increasing ATP/ADP to close ATP-dependent K+ (KATP) channels, and in turn, depolarizing the β-cell membrane potential. This results in the opening of voltage-gated calcium channels, promoting the influx of calcium and secretion of an immediately releasable pool of granules (15, 37, 46). Second-phase secretion consists of a sustained elevation of insulin secretion, which rises over a period of several minutes (2, 8, 20, 45) and is typically accompanied by insulin pulses occurring at 5- to 15-min intervals (34).

This description of biphasic insulin secretion is typical of nearly all species, whether measured from in vivo plasma concentrations (6, 7), perfused pancreas (8, 12), or perfused islets in vitro (21). A notable exception is the isolated mouse islet, which displays a small and relatively static second phase after a fairly typical first-phase response. This type of flat second phase has been observed using a variety of mouse preparations, including the perfused pancreas and cultured islets, and is seen in several different mouse strains (4, 13, 23, 26, 42, 56, 57).

To determine whether second-phase secretion in the mouse is the result of a physiological difference between mice and other species or results from the removal of the pancreas from its native environment requires the direct measurement of insulin from mice in vivo. To date, a detailed secretory profile of insulin release from conscious mice has been lacking because of technical challenges with surgical procedures, glycemic clamping of small and delicate blood vessels, and maintaining sufficient blood volume to permit rapid sampling of plasma. We have overcome these limitations and report the successful use of a hyperglycemic clamp in conscious mice to assess first-phase, second-phase, and pulsatile patterns of insulin secretion. We thus demonstrate a new technique that will allow investigators to measure patterns of insulin secretion in various transgenic mouse models.

MATERIALS AND METHODS

Male Swiss-Webster mice (20–35 g) were used for all experiments using protocols in accordance with the Animal Care and Use Committee of Virginia Commonwealth University, Vanderbilt University, or the University of Washington, consistent with National Institutes of Health guidelines. Unless otherwise stated, all chemicals used in these experiments were obtained from Sigma-Aldrich (St. Louis, MO).

Measuring insulin and glucose concentration in conscious mice.

The surgical procedures used were similar to those described previously (14, 30, 38). Mice were anesthetized with pentobarbitol sodium (70 mg/kg body wt). The left common carotid artery was catheterized (70 mg/kg body wt). The left common carotid artery was catheterized (70 mg/kg body wt). The right jugular vein was catheterized for infusions with a Silastic catheter (0.025 in. OD). The free ends of the catheters were tunneled under the skin to the back of the neck, where they were attached via stainless steel connectors to lines made of Micro-Renathane (0.033 in. OD), which were exteriorized and sealed with stainless steel plugs. Lines were flushed daily with 10–50 μl of saline containing 200 U/ml heparin and 5 mg/ml ampicillin. Animals were individually housed, and body weight was recorded daily for 5–7 days after surgery.

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Food was withheld from each mouse at 5:00 PM on the day before an experiment. On the morning of each study, conscious mice were placed in a 1-liter plastic container lined with bedding, and the Micro-Renathane tubing (22 cm long, 0.033 in. OD) was connected to the catheter leads. After ~1 h, two blood samples were taken 5 min apart to measure baseline glucose and insulin. Next, a variable infusion of 50% dextrose solution was infused in the jugular vein to increase the glucose to 200–250 mg/dl (mean: 230 ± 8 mg/ml among all 11 mice). This infusion produces a glucose concentration of ~13 mM in the mouse compared with basal glucose levels of ~5 mM. One of two protocols was then implemented to collect blood samples. For protocol 1, a rapid-sampling period was initiated immediately after dextrose infusion to assess both first- and second-phase insulin secretion (n = 6 mice). Blood samples (60 μl) were collected every minute for 20–30 min (in the case of one mouse, sampling was made at 2-min intervals). For protocol 2, blood collection for insulin measurements was delayed for 40–60 min to assess pulsatility under conditions of elevated, steady-state glucose (n = 5 mice).

To avoid decreases in blood cell volume from sampling, a constant supply of blood (70 μl/min) was infused from a donor mouse throughout the sampling period for both protocols. Replacement of red blood cells at a constant rate is more effective and less disruptive to the mouse than replacement of red cells as a bolus. Although we cannot rule out that this constant infusion can, in principle, affect the neuroendocrine responses to hyperglycemia, we have observed that this procedure does not increase plasma catecholamines (3).

Blood was collected from the same arterial catheter to measure glucose and insulin. Glucose was measured using a HemoCue B-Glucose Analyzer (HemoCue, Lake Forest, CA), which accurately measures blood glucose within a range of 0–400 ± 6 mg/dl using 5 μl of blood. Immunoreactive insulin was assayed in duplicate using a double-antibody method (28) with reagents obtained from Linco Research (St. Charles, MO). The intra- and interassay variabilities for insulin were 6–10% and 4–7%, respectively.

**Insulin secretion from dissociated islets.** Mice were killed, and islets were isolated by collagenase digestion from the mouse pancreas, as previously described (47). RPMI-based medium was replaced with a low-glucose Krebs solution containing (in mM) 2.8 glucose, 102 NaCl, 5 KCl, 1.2 MgCl₂, 2.7 CaCl₂, 20 HEPES, 5 NaHCO₃, and 1 mg/ml BSA, pH 7.4. Islets were incubated for 60 min to allow sufficient time for adjustments in metabolism to occur. After 60 min, samples were collected at 2-min intervals for 10 min, and then the low-glucose solution was removed and replaced with a high-glucose solution containing (in mM) 11.1 glucose, 132 NaCl, 5 KCl, 1.2 MgCl₂, 1 CaCl₂, 10 HEPES, 5 NaHCO₃, and 1 mg/ml BSA, pH 7.3. Samples were then collected at 2-min intervals for the first 10 min and then at 5-min intervals for the next 20 min. Insulin was measured as previously described (39, 51).

It should be noted that, although a similar relative shift of ~8 mM glucose was used for in vitro and in vivo experiments, the basal and stimulated glucose concentrations differed between in vivo (~5 and ~ 13 mM) and in vitro (2.8 and 11.1 mM) studies. Because different tissue preparations (whole animal, perfused pancreas, isolated islets, or dispersed β-cells) can display different thresholds for glucose activation (17, 52), we experimentally determined that a step from 2.8 to 11.1 mM glucose produced basal and first-phase secretory responses that were qualitatively and quantitatively similar to the in vivo findings compared with other glucose concentrations (using 5

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**Fig. 1.** Second-phase insulin secretion differs between conscious mice in vivo and mouse islets in vitro. A and B: examples of insulin measurements demonstrating a biphasic response from conscious mice in vivo (A) and from groups of perfused islets in vitro (B). Dotted lines indicate the mean basal insulin levels measured before glucose was increased.
mM glucose as a basal concentration, for example, generated a weak first-phase response). This strategy, we hoped, would provide a more accurate assessment of second-phase differences. Previous studies have established that mouse islets demonstrate a small and static second phase under a variety of different glucose challenges (4, 13, 23, 26, 42, 56, 57), so our choice of glucose concentrations may not have mattered. For example, shifting glucose from 1.66 to 11.1 mM in two groups of islets still resulted in a small, static second phase (153 ± 50) following a substantial first-phase response (1,112 ± 137%). Because of the large first-phase response, data from these two groups were used in calculating the slope of second-phase secretion only.

Statistics and pulse detection. First-phase peak and second-phase plateau were expressed as a percentage of basal insulin to permit a direct comparison between insulin concentrations measured in the whole mouse vs. insulin secretion from perfused islets. First- and second-phase secretion comparisons between mice and perfused islets were made using the Mann-Whitney test because of the non-parametric nature of the data, although the data were also found to be significant using a two-tailed t-test assuming unequal variance. The rate of second-phase increase for mice and perfused islets was determined by linear regression, and the slopes were compared with a slope of zero to determine statistical significance. Pulses of insulin were identified with the pulse detection algorithm Cluster (31, 50) using the following parameters: 1-min minimum peak size, 1-min minimum nadir size, 2.0 for t-score to detect peaks and nadirs, and no minimum value for peak amplitude. False positives were kept to <5% by using the intra-assay variance as a SD for each data point of a given record of insulin concentration. To improve the detection of possible false negatives, the data were reanalyzed using a t-score of 1, producing the same results.

RESULTS

Biphasic insulin secretion in conscious mice and perfused mouse islets. Because mouse islets display an atypically flat second phase compared with the islets of most other species, the goal of this study was to determine if insulin secretory patterns measured in vivo in conscious mice similarly lack second-phase secretion. As shown in Fig. 1, insulin levels increased sharply and transiently before falling to a second-phase plateau above basal secretion in both conscious mice and islets in vitro in response to increased glucose. The second phase shown in the two in vivo examples in Fig. 1A, however, continually rose throughout the experiment, in contrast to the relatively flat second phase observed for groups of ~75 perfused mouse islets measured in vitro (Fig. 1B), indicating that mice indeed display a second phase of secretion in vivo.

The mean values of in vivo insulin during basal, first-phase peak, and second-phase insulin secretion were 98.7 ± 20.8, 346.1 ± 57.1, and 263.1 ± 46.7 pmol/l, respectively (n = 5). A summary of biphasic secretion comparing in vivo and in vitro findings is shown in Fig. 2. Both first and second phases are represented in Fig. 2A as a percentage of basal secretion measured before the glucose challenge. First-phase secretion for mice (403 ± 73%, n = 5) did not significantly differ (P > 0.41) from the first phase of insulin secretion for isolated mouse islets in vitro (508 ± 94%, n = 4).

Second-phase secretion, in contrast, differed significantly (P < 0.05) between conscious mice (289 ± 41%, n = 5) and mouse islets (169 ± 9%, n = 4), as evidenced by the mean percent increase over basal secretion during exposure to 11.1 mM glucose. In addition, the trajectory of the secretory profiles differed, with a continuously rising second phase observed in mice (slope: 2.63 ± 0.39%/min, P < 0.01 against a slope of 0), compared with the second phase seen in mouse islets in vitro (slope: 0.18 ± 0.14%/min, P > 0.30, not statistically different from a slope of 0, n = 6). This is shown in Fig. 2B in which insulin profiles were normalized to the first-phase peak and presented as an average of five mice and four groups of mouse islets.

To ensure that the rising second phase we observed in vivo was not the result of inadequate glycemic clamp, glucose measurements were compared between the first 5 min and the last 5 min of the second-phase response for each mouse. In four of five mice, the increase in mean glucose from the beginning of the second phase to the end of the experiment was only 6 ± 10%, whereas insulin concentration rose 76 ± 27% during the second phase for these same four mice. The remaining mouse was less adequately clamped, with glucose at the end of the experiment rising 102%, although the increase in insulin was even greater, rising 216% from beginning to end. These data thus indicate that insulin concentration increased during the second phase even when glucose concentrations were well clamped.
Pulses in insulin during second-phase secretion. A more detailed analysis of the insulin patterns observed during second-phase secretion revealed distinct pulses in 9 of 11 mice. Our earliest experiment involved measuring insulin at 2-min intervals, as shown in Fig. 3A. Two pulses in Fig. 3A were detected at 8-min intervals using Cluster analysis, although two additional smaller pulses could be detected only when the pattern was analyzed assuming no SD in the sampling, yielding a 4.7 ± 0.7-min pulse interval. We stress that these two minor pulses could not be detected using the appropriate parameters for Cluster analysis and suggest more rapid sampling was needed. Thus insulin secretion was measured at 1-min intervals in all subsequent experiments. The example in Fig. 3B demonstrates clearly defined pulses at 4.5 ± 0.5-min intervals.

We also determined that the insulin patterns remained oscillatory well after the initial glucose challenge. Figure 3C shows the glucose concentration for the full 83-min sampling period and plasma insulin for the last 13 min. Two basal readings of both insulin and glucose were taken before dextrose infusion. The glucose concentration doubled in the mouse within minutes, but blood sampling for insulin was delayed an additional 60 min, since only enough blood was available to take 10–20 samples. Pulsatile insulin secretion continued, as demonstrated by the two prominent peaks observed in Fig. 3C measured 1 h after the initial increase in blood glucose (representative of experiments in 5 mice). Glucose sampling confirmed that the glucose concentration was stable in the mouse during this time period.

The pulse characteristics of peak interval, width, peak height as percent above baseline, and area are summarized in Table 1 for all nine mice in which pulsatile insulin was detected. No differences were observed between protocols 1 and 2 in any pulse characteristics (P ≥ 0.35, n = 4 for each protocol). For the two mice in which pulses of insulin secretion were not detected, insulin was collected using protocol 1 for one mouse and protocol 2 for the other mouse. Mean values for each pulse characteristic shown in Table 1 were obtained using records from the eight mice in which insulin was sampled at 1-min intervals (mouse 102–04 was excluded because of the different sampling rate).

**DISCUSSION**

It has been suggested that first-phase insulin secretion is mediated by the “consensus K<sub>ATP</sub> pathway,” whereas the second phase is mediated by another mechanism, variously referred to as the “K<sub>ATP</sub> channel-independent pathway” (41, 44), the “nonionic pathway” (2), and the “amplifying pathway” (16). This second-phase pathway was first identified in mouse islets (10, 11) and rat islets (40). Subsequent comparative studies between mouse and rat islets have established that stimulation of this pathway is less robust in mouse islets in vitro (4, 23, 26, 42, 56). This finding has led to several reports investigating the mechanisms underlying the different degrees of stimulation of second-phase secretion in mouse and rat islets.

**Table 1. Insulin pulse characteristics**

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Interval, min</th>
<th>Width, min</th>
<th>Peak, %basal</th>
<th>Area, pg/ml 10 min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>102–04*</td>
<td>8</td>
<td>4.0</td>
<td>158</td>
<td>1.15</td>
</tr>
<tr>
<td>124–04</td>
<td>4.0</td>
<td>2.7</td>
<td>156</td>
<td>0.72</td>
</tr>
<tr>
<td>137–04†</td>
<td>5.0</td>
<td>4.3</td>
<td>205</td>
<td>2.33</td>
</tr>
<tr>
<td>138–04‡</td>
<td>6.0</td>
<td>4.5</td>
<td>130</td>
<td>0.97</td>
</tr>
<tr>
<td>175–04†</td>
<td>3.0</td>
<td>1.8</td>
<td>144</td>
<td>0.74</td>
</tr>
<tr>
<td>176–04‡</td>
<td>3.5</td>
<td>3.0</td>
<td>174</td>
<td>1.27</td>
</tr>
<tr>
<td>183–04</td>
<td>4.5</td>
<td>3.2</td>
<td>329</td>
<td>3.50</td>
</tr>
<tr>
<td>184–04</td>
<td>3.0</td>
<td>2.2</td>
<td>185</td>
<td>1.00</td>
</tr>
<tr>
<td>193–04</td>
<td>4.5</td>
<td>4.3</td>
<td>166</td>
<td>3.51</td>
</tr>
</tbody>
</table>

Mean ± SE 4.2 ± 0.4 3.2 ± 0.3 181 ± 20 1.92 ± 0.61

*Pulses detected at 2-min intervals. Data are not included in the calculation of means. †Insulin collected 40–60 min after increasing plasma glucose level.
islets, including glyceraldehyde (23), cAMP (26), protein kinase A (48), phospholipase C (53, 56), and amino acids (25, 55) and has even led to speculation that the mouse may not be a good model for examining insulin secretion as it relates to humans, which have a robust second phase (26).

In this report, we provide a detailed description of insulin secretory patterns in conscious mice. Our findings suggest that, as in other species, mice respond to a sharp increase in glucose with biphasic insulin secretion, specifically with a more robust second-phase response than suggested by in vitro studies (4, 23, 26, 42, 56). Although our findings do not address the intracellular pathways involved in second-phase secretion, our findings do indicate that insulin secretion at the whole animal level is likely to be more similar to that of other species. This suggests that, in mice, an important input in vivo to the pancreas or to the islets might be lost after islet isolation. This input may consist of nervous innervation of the pancreas [1 or important circulating factors, such as incretins (9a)]. Islet isolation and culturing may also cause changes in gene expression (18, 24, 54) or insulin granule dynamics (37, 45, 46) that do not occur to the same extent in islets from other species. Future studies will be required to more fully address these possibilities.

It should be noted that insulin absorption and degradation by the liver, which can be up to 80% in humans (27) and ~50% in dogs (19, 33), as well as arterial blood circulation, are confounding factors in any attempt at a direct comparison of in vivo and in vitro studies. Our comparisons were thus based on percentage changes within the patterns of insulin secretion from mice and within patterns of insulin secretion from perfused islets. Although it can be argued that such comparisons are suspect nonetheless, the key finding of a rising second phase in insulin secretion in vivo, but not in vitro, was also validated by comparing the slopes of second-phase secretion independently of one another against a slope of zero. This demonstrated that the trajectory of mouse insulin secretion was clearly rising and the trajectory of mouse islets was statistically flat.

We also observed that, as in other species, mouse insulin secretion is pulsatile. Although it should be noted that a small subset of pulsatile insulin data was published elsewhere (32), the more detailed analysis in the present study demonstrates that mouse insulin patterns occur with a period of ~4 min. Although we attempted to assess pulses as accurately as possible (see MATERIALS AND METHODS), there are limitations to pulse analysis that can result in the detection of false positive and/or false negative pulses (34, 43). By analyzing records as short as 20–30 min, we increase the possibility of errors in accurately measuring pulse characteristics. It should be stressed, however, that these studies are technically difficult. For example, to generate a 30-min record of insulin secretion, ~2 ml of blood must be collected, which is nearly the total blood volume of the mouse. Although we used blood from a donor mouse to maintain overall red blood cell volume (see MATERIALS AND METHODS), continuing to take additional samples beyond 30–40 min would begin to reduce the red blood cell volume, which could begin to adversely affect the mouse. Although a longer duration of insulin secretion from the mouse would thus be preferable for evaluating secretory patterns, this is not presently feasible.

Despite these caveats, pulsatile secretion is observed in other species and thus not unexpected in mice. We observed robust and regular patterns of pulsatile insulin in several mice at similar intervals ranging from ~3–6 min, as assessed by visual inspection in addition to rigorous pulse analysis, suggesting these are valid pulse intervals for the mouse. Although insulin pulses have been reported to have periods of 5–15 min in other species (see review in Ref. 34), recent studies using improved sampling and analysis techniques suggest that insulin pulses may actually occur at intervals nearer to 5 min in rodent species (22, 35) and in humans (36, 43). Of interest, a pulse interval of 3–5 min is consistent with the period of rhythms in mouse islet fuel metabolism, electrical activity, intracellular calcium, and secretion measured in vitro (5, 9, 31, 49, 58).

In conclusion, this study confirms that mice respond to increased glucose with a first-phase peak and subsequent insulin pulses, as do other species. The robust second-phase observed in conscious mouse contrasts with the small, flat second phase reported in mouse islets in vitro and confirmed in this report, suggesting that an important physiological mechanism needed to generate second-phase insulin secretion may be diminished or lost in dissociated mouse islets in vitro. At the level of the animal, however, insulin secretion in the conscious mouse displays phases and pulses similar to those of other species. This study thus validates mice as a model for measuring insulin secretion and opens the door for investigators to study insulin secretion in various transgenic mouse models.

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